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Application of Synthetic Liposomes Based on Acyl Amino Acids or Acyl Peptides as Drug Carriers. I. Their Preparation and Transport of Glutathione into the Liver

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Palmitoyl amino acids and palmitoyl glutathione were synthesized. Liposome-like vesicles based on these compounds were prepared and their barrier functions were examined. These vesicles showed encapsulation efficiencies for aqueous solute comparable to that of conventional phosphatidylcholine liposomes (PC-liposomes). They were also stable in fresh rat plasma at 37°C.

The biological behavior (blood clearance, urinary excretion and tissue distribution) of the vesicles based on palmitoyl serine (PSer-liposomes) or palmitoyl glutathione (PGSH-liposomes) was examined after intravenous injection in rats. The synthetic liposomes were cleared very rapidly from the blood compared with PC-liposomes. PSer-Liposomes showed a large amount of urinary excretion of aqueous marker (³Hjninulin), suggesting that the mechanisms of vesicle degradation *in vivo* and *in vitro* are different. These synthetic liposomes showed low affinity to the spleen and high affinity to the liver in the tissue distribution study, and thus they may be expected to be a useful drug carrier which is targetable to the liver.

A suppressing effect of PGSH-liposomes on the increase of plasma glutamate oxaloacetate transaminase (GOT) induced by a high dose of acetaminophen in mice was observed, and transport of glutathione into the liver cells apparently occurred. The suppressing effect was greater than that of free glutathione or PC-liposomes containing free glutathione. However, the effect was not observed in the case of PGSH-liposomes without phosphatidylcholine, which appears to have an important role in the liposome-cell interaction.

Keywords—liposome; drug carrier; palmitoyl serine; palmitoyl glutathione; stability; tissue distribution; targeting; acetaminophen; liver necrosis

Introduction

In a previous report,²⁾ it was shown that synthetic alkyl glycosides formed lamellar vesicles like phosphatidylcholine vesicles (liposomes), and the preparation and physical properties of these vesicles were described. The targeting efficiency of the vesicles based on palmitoyl galactoside to the liver was also reported.³⁾ Application of synthetic acyl amino acids and acyl peptides was attempted in the present study, and the preparation of the vesicles and glutathione-transporting ability of the acyl glutathione vesicles are described here.

Yatvin *et al.*⁴⁾ reported pH-sensitive liposomes composed of synthetic palmitoyl homocysteine. This is an example of application of an acyl amino acid as a liposomal component. If acyl amino acids form lamellar liposome-like vesicles, as alkyl glycosides do, they could be useful for the development of effective drug carriers or functional liposomes, because amino acids have various functional groups and it should be possible to carry out chemical modifications on the liposomal surface. Neuman and Ringsdorf⁵⁾ prepared liposomes composed of amino acids coupled with a long hydrocarbon chain. They attempted to stabilize the liposomes by the formation of amide bonds on the surface of the liposomes.

In this report, preparation of liposomes based on *N*-palmitoyl amino acids and the encapsulation capacities of the liposomes were investigated, and the stability in plasma and disposition *in vivo* of palmitoyl serine liposomes were examined as an example.

Many applications of lipophilized peptides which are biologically active, such as muramyl dipeptide (MDP), have recently been attempted.⁶⁾ Liposomes injected *in vivo* were phagocytosed by macrophages and the peptide then activated the macrophages. We focussed on glutathione, which has an important role in detoxication in the liver.⁷⁾ Glutathione is not readily taken up by the liver in free form,⁸⁾ but esters of glutathione⁹⁾ are taken up more effectively. Anderson *et al.*¹⁰⁾ studied the uptake of glutathione monoethyl ester by tissues and its conversion to glutathione. On the other hand, it is known that substantial fractions of intravenously injected liposomes are taken up by the liver,¹¹⁾ probably mainly by the Kupffer cells. However, small liposomes such as small unilamellar vesicles (SUV) may pass through the fenestration of the liver sinusoids and could reach the parenchymal cells.^{12,13)} Malnoe *et al.*¹⁴⁾ studied the effect of liposomal entrapment on the protective action of glutathione against acetaminophen-induced liver necrosis. Wendel *et al.* reported¹⁵⁾ the transport of glutathione by liposomes encapsulating glutathione in the aqueous phase, but a part of the aqueous contents was apparently released very rapidly in the blood.^{16,17)} Further, the multilamellar vesicles (MLV) that they used may not be able to be taken up by the parenchymal cells, because they cannot pass through the fenestration and thus cannot reach the cells.^{12,13)} The use of small liposomes might not improve the situation, because their capacity for glutathione transport would be small.

In this study, we attempted to transport glutathione into the hepatocytes by using small vesicles composed of acyl glutathione. The effectiveness of transport was assessed in terms of the suppressing effect on the increase of plasma glutamate oxaloacetate transaminase (GOT) induced by a high dose of acetaminophen.¹⁵⁾ The stability in plasma and disposition *in vivo* of the palmitoyl glutathione liposomes were also examined.

Experimental

Materials—Palmitic acid, L-serine and L-lysine were purchased from Nakarai Chem. Ltd. (Kyoto). Glycine, L-cystine and L-glutamic acid were from Kanto Chem. Co., (Tokyo). *N*-Hydroxysuccinimide and dicyclohexylcarbodiimide were of special grade for peptide synthesis, and were purchased from Nakarai Chem. Ltd. 5(6)-Carboxyfluorescein (Kodak, Rochester, N.Y.) was used without purification. Hydrogenated egg-phosphatidylcholine and glutathione were gifts from Nippon Fine Chem. Co., (Osaka) and Yamanouchi Pharm. Co., (Tokyo), respectively. All other chemicals and reagents were as described in the previous papers.^{2,3)}

Synthesis of *N*-Palmitoyl Amino Acids—*N*-Hydroxysuccinimide ester of palmitic acid (PS) was prepared and crystallized (yield 67.5%, mp 90 °C) as described by Lapidot *et al.*¹⁸⁾ This active ester was reacted with amino acids (L-serine, L-lysine, L-cystine, L-glutamic acid and glycine) in the manner described by Lapidot *et al.*¹⁸⁾ Yield (Y) and melting point (mp) of obtained palmitoyl amino acids were as follows: palmitoyl glycine (PGly, Y 27.3%, mp 120 °C); palmitoyl serine (PSer, Y 52.3%, mp 95–97 °C); dipalmitoyl cystine (PCys–CysP, Y 64.0%, mp 105 °C); dipalmitoyl lysine (PlysP, Y 59.2%, mp 101–103 °C); palmitoyl glutamic acid (PGlu, Y 15.8%, mp 110–111 °C).

Synthesis of *N*-Palmitoyl Glutathione—Glutathione (GSH) was oxidized by air in alkaline solution (NaHCO₃) at 40 °C by the method of Aoyagi *et al.*¹⁹⁾ Oxidized glutathione (GSSG) was coupled with palmitic acid in the same manner as used for amino acids, as described above. Oxidized palmitoyl glutathione (PGSSGP) was reduced with NaBH₄. The reaction mixture was adjusted to pH 1 with 10% HCl, then condensed. Water was added to the residue and the mixture was filtered. The product was recrystallized from MeOH–H₂O several times. Palmitoyl glutathione (PGSH) was obtained in 49.8% yield (mp 170–171 °C). These compounds were checked by infrared (IR) spectrometry, mass spectrometry and elemental analysis. The results of elemental analyses are shown in Table I.

Preparation of Liposomes—Liposomes were prepared by the conventional lipid-film-hydration method.²⁾ In the case of lipid with low solubility in CHCl₃, MeOH was used as the solvent. The lipid composition of the liposomes was palmitoyl residue of palmitoyl amino acid or palmitoyl glutathione and cholesterol (CH) in a molar ratio of 2 : 1 and the lipid concentration was 60 μmol/ml as total lipids. Liposomes used for biological experiments were sonicated with a bath-type sonicator (Tocho IUC-2811, Tokyo) for 2 h at 0 °C after preparation as above. [¹⁴C]Sucrose and 5(6)-carboxyfluorescein (CF) were used as aqueous markers in the encapsulation study and stability study.

TABLE I. Elemental Analyses of Synthetic Palmitoyl Compounds

Compounds	Found (%)			Calc (%)		
	C	H	N	C	H	N
PSer·H ₂ O	63.15	10.91	3.94	63.16	10.80	3.88
PGly	68.75	11.40	4.50	69.01	11.18	4.47
PGlu	60.59	9.41	3.32	60.50	9.35	3.64
PCys-CysP	63.26	10.19	3.77	63.69	10.06	3.91
PLysP	72.04	11.89	4.58	73.31	11.90	4.50
PGSH	56.78	8.85	7.01	56.83	8.37	7.65

respectively. The fluorescent marker (CF) provides an immediate and easily measurable index of membrane permeability and has been widely used for stability studies.²⁰⁾ Unencapsulated marker was removed by dialysis in cellulose tubing. [³H]Inulin was used as a marker in the *in vivo* behavior study, and dialysis was carried out in a flow-type dialysis cell with a polycarbonate membrane (Nucleopore Co., CA) having a pore size of 0.05 μ m as presented in the previous paper.²¹⁾ Inulin is considered to be a suitable liposomal marker for *in vivo* disposition studies, because it is recognized to be biologically inactive and excreted rapidly and completely.¹⁷⁾

Stability in Plasma—A 0.1 ml aliquot of the liposome (sonicated) suspension containing 100 mM CF was incubated with 0.9 ml of fresh rat plasma at 37 °C. After appropriate time intervals, a 10 μ l aliquot of the incubation mixture was transferred into a tube containing 5.0 ml of cold phosphate-buffered saline (PBS). A 1 ml aliquot of the diluted mixture was treated with Triton X-100. The latency of the liposomes was calculated from the fluorescence intensities with and without Triton X-100 treatment.

Blood Concentration and Urinary Excretion—Male albino Wistar rats (body weight; 250 \pm 20 g) were cannulated in the femoral vein, femoral artery and bladder, and treated as described in the previous paper.¹⁷⁾ A 0.5 ml aliquot of the liposome suspension (30 μ mol total lipids) was injected through the cannula inserted into the femoral vein. At appropriate times after injection, arterial blood and urine samples were collected and the radioactivity of [³H]inulin was counted as described in a previous paper.¹⁷⁾

Tissue Distribution—Immediately after the last sampling of blood and urine (4 h after injection), the animal was sacrificed and the liver, lungs, spleen and kidneys were isolated. The remaining radioactivity of [³H]inulin in each organ was counted as described in a previous paper.²¹⁾

Transport of Glutathione into the Liver—Transport of glutathione into the liver was examined in terms of the suppressing effect on the increase of plasma GOT induced by a high dose of acetaminophen.¹⁵⁾ Male ddY mice (body weight; 25–30 g) were given acetaminophen intraperitoneally at the dose of 500 mg/kg in dimethylsulfoxide solution (250 mg/ml). More acetaminophen (same dose) was injected at 1.5 h after the first injection in order to confirm the increase of plasma GOT. Various preparations of sonicated liposomes were injected intravenously at 30 min before the first injection of acetaminophen. The animal was sacrificed by puncturing the jugular vein under light anesthesia with ether at 24 h after the second acetaminophen injection. The blood was sampled rapidly with a heparinized pipet. The activity of plasma GOT was determined by the method of Reitman and Frankel²²⁾ and expressed in terms of the absorbance of 2,4-dinitrophenylhydrazone at the wavelength of 505 nm.

Results and Discussion

Characterization and Encapsulation Capacity of Liposomes

Optical micrographs of palmitoyl serine liposomes (PSer-liposomes) and palmitoyl glutathione liposomes (PGSH-liposomes) are shown in Fig. 1. Spherical liposomal structures were observed. Liposomes composed with other palmitoyl amino acids showed similar spherical structure.

The values of encapsulation ratio of aqueous [¹⁴C]sucrose solution in the liposomes calculated from the radioactivity of the suspension after and before dialysis are shown in Table II. Liposomes based on palmitoyl amino acid or palmitoyl glutathione showed an encapsulation capacity for aqueous marker that was comparable to that of phosphatidylcholine liposomes (PC-liposomes) with cholesterol. On the other hand, they showed no encapsulation capacity without cholesterol. This result indicates that these compounds form a lamellar structure encapsulating an aqueous phase, like liposomes, when cholesterol is

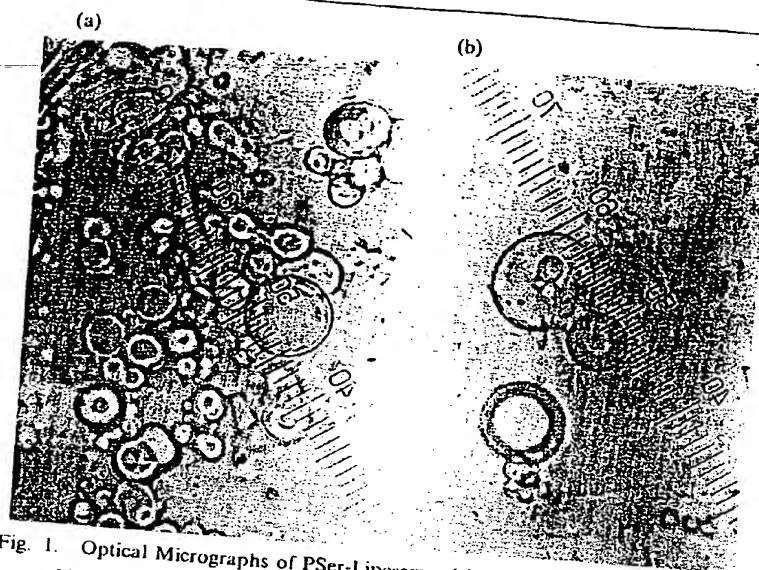


Fig. 1. Optical Micrographs of P-Ser-Liposomes (a) and PGSH-Liposomes (b). Liposomes were prepared by the lipid-film-hydration method and were composed of palmitoyl compound (as palmitoyl residue) and cholesterol in a molar ratio of 2:1 (1 div. = 1.67 μ m).

TABLE II. Encapsulation Ratios of [14 C]Sucrose Entrapped in Liposomes Based on Synthetic Palmitoyl Amino Acids and Palmitoyl Peptides

Base	Encapsulation ratio (%)	
	With CH	Without CH
PC	17.4 \pm 2.4	—
P-Ser	13.0 \pm 1.8	0.02
PGly	18.8 \pm 1.5	0.03
PCys-CysP	13.3 \pm 2.4	0.03
PLysP	15.0 \pm 3.4	0.05
PGlu	18.8 \pm 1.3	0.02
PGSH	14.9 \pm 2.9	0.04
PGSSGP	12.8 \pm 2.6	0.18

Lipid composition was base (as palmitoyl residue) and cholesterol (CH) in a molar ratio of 2:1. Values are expressed as means \pm S.D. of three experiments.

present. However, they do not form such a structure without cholesterol. Therefore, the lamellar structure was suggested to be stably constructed with alternate hydrophobic anchors of palmitoyl chain and cholesterol in the lipid layer.

In the case of sonicated liposomes, their encapsulation ratios were about 1%, comparable to that of sonicated PC-liposomes. Very small particles were observed by optical microscopy. The palmitoyl compounds seem to form very small liposomes and retain their barrier function even after sonication. Small liposomes are advantageous for interaction with the hepatocytes because they are able to pass through the fenestration of the liver sinusoids,^{12,13)} and therefore the sonicated liposomes were used in the following experiments.

Stability in Plasma

It is known that liposomes are destroyed in the blood and release the entrapped aqueous

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contents.^{16,17)} For the application of the vesicles described here as drug carriers *in vivo*, they must be stable in plasma. The stabilities of the sonicated PSer-liposomes and PGSH-liposomes in fresh plasma were examined and compared with that of PC-liposomes. The results are shown in Fig. 2 as the time course of latency during incubation with fresh rat plasma at 37°C. PC-Liposomes showed gradual degradation followed by a plateau latency of 85%. On the other hand, PSer-liposomes were very stable and showed little leakage during incubation for 2 h. They showed a leakage of about 6% even at the first sampling time (1.5 min), but this is considered to be due to the adsorbed dye on the liposome surface or very rapid leakage because of temperature shock at the initiation of the incubation, or some other such mechanisms. PGSH-Liposomes showed a degradation profile similar to that of PC-liposomes and they are considered to have comparable stability to PC-liposomes in plasma.

Clearance from the Blood

Clearance of the sonicated liposomes from the blood after intravenous injection is shown in Fig. 3. PC-Liposomes were retained in the blood for a long time, and radioactivity amounting to about 2.4% of injected dose was observed in 1 ml of the blood even at 4 h after the injection. This value corresponds to about 50% of dose in whole blood (blood volume was estimated as 8% of body weight). On the other hand, PSer-liposomes and PGSH-liposomes were cleared rapidly and little radioactivity was observed in the blood even at 1 h after injection. In particular, PSer-liposomes were cleared very rapidly and only 1.9%/ml of the injected radioactivity was observed at 3 min after the injection. It is known that the clearance of liposomes is affected by particle size.¹⁷⁾ The size of particles used in this experiment were not determined, but there should not be much difference between PC- and palmitoyl-compound-liposomes because they were prepared by the same procedure and the particle sizes were confirmed to be very small by optical microscopy. Therefore, it is reasonable to consider that the clearance mechanisms of the PC-liposomes and synthetic liposomes presented here may be different.

Excretion of an Aqueous Marker in Urine

Cumulative radioactivity excreted in urine after intravenous injection of the liposomes is presented in Fig. 4 as a percentage of the injected dose. The initial increasing phase in the

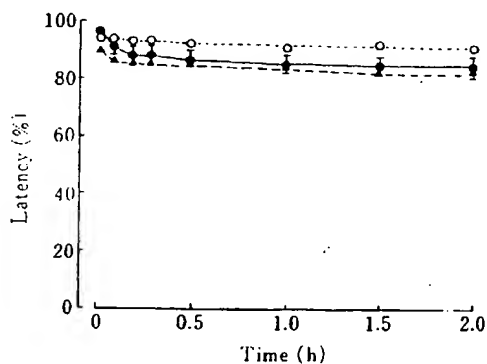


Fig. 2. Stability of PC-Liposomes (—●—), PSer-Liposomes (---○---) and PGSH-Liposomes (---▲---) in Plasma

Liposomes were prepared by the sonication procedure and their lipid compositions were the same as those in Fig. 1. Latency was calculated from the fluorescence intensity of CF lost from the liposomes during incubation with fresh rat plasma at 37°C.

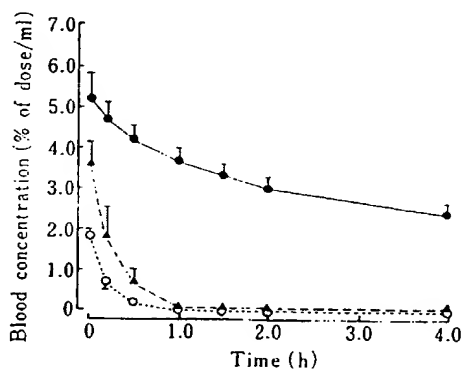


Fig. 3. Time Courses of Blood Levels of [³H]-Inulin Entrapped in Liposomes after Intravenous Injection into Rats

Liposome preparations and symbols are the same as those in Fig. 2. Blood concentration was determined based on the radioactivity of [³H]inulin used as an aqueous space marker. The dose was 30 μ mol/head as total lipids.

cumulative excretion curve may represent the rapid leakage of the aqueous marker, [^3H]inulin, from the liposomes in the circulation as described in the previous paper.¹⁷⁾ PSer-Liposomes showed a large excretion in the initial phase, even though the *in vitro* stability study-in-fresh plasma showed that the liposomes were very stable, as mentioned previously. This result suggests the possibility that the *in vivo* stability in the early stage after the injection is affected by some other factor(s) than those existing in plasma, e.g. turbulence of the blood flow, interaction with cells in the blood or tissues, etc. PC-Liposomes and PGSH-liposomes seemed to be resistant to these factor(s) and showed lower urinary excretion.

Tissue Distributions

Remaining radioactivity in each organ at 4 h after injection is listed as a percentage of the injected dose in Table III. It is known that intravenously injected liposomes accumulate in the liver and spleen.¹¹⁾ In the present experiment, 23.6% of dose was found in the liver and 8.7% in the spleen after injection of PC-liposomes. Little radioactivity was found in the kidneys and lungs (less than 1%). In the case of PSer-liposomes, 30.1% and 1.8% of the dose were found in the liver and spleen, respectively. Radioactivities in the kidneys and lungs were very low (<0.4%). The most remarkable difference between PC-liposomes and PSer-liposomes was in the accumulation in the spleen. PSer-Liposomes showed less accumulation than PC-liposomes (about one-fifth of that of PC-liposomes). The tissue distribution pattern of PGSH-liposomes was very similar to that of PSer-liposomes except for the liver. PGSH-liposomes accumulated in the liver more markedly than PSer-liposomes (67.4% of dose).

The results obtained in this experiment indicated that the liposomes based on palmitoyl amino acid or palmitoyl peptide are selectively accumulated in the liver, not in the spleen, and their blood concentration is very low after intravenous administration. Though PSer-liposomes are less stable in the circulation and their aqueous contents are rapidly excreted in the urine, the synthetic liposomes described here may be useful for targeting to the liver. It is

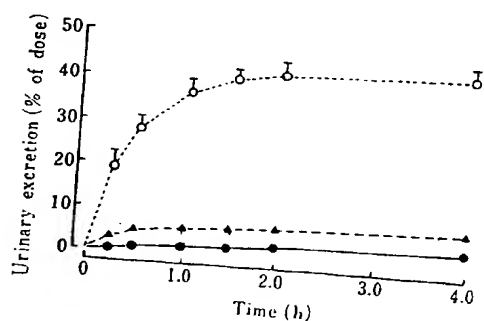


Fig. 4. Cumulative Excretion of [^3H]inulin in Urine after Intravenous Injection of [^3H]inulin Entrapped in Liposomes into Rats
Liposome preparation, symbols and method of determination are the same as those in Fig. 3.

TABLE III. Tissue Distribution of [^3H]inulin Entrapped in Liposomes after Intravenous Injection into Rats at the Dose of $30 \mu\text{mol/head}$

Organ	Distribution (% of dose)		
	PGSH	PSer	PC
Liver	67.42 ± 2.47	30.14 ± 2.65	23.57 ± 4.73
Kidney	0.33 ± 0.08	0.36 ± 0.04	0.58 ± 0.15
Lung	0.31 ± 0.07	0.37 ± 0.04	0.76 ± 0.25
Spleen	1.91 ± 0.31	1.83 ± 0.48	8.71 ± 3.07

Values are expressed as mean \pm S.D. of four experiments.

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TABLE IV. Effects of Glutathione in Various Dosage Forms against Plasma GOT Induction by Acetaminophen in Mice

Dosage form	Dose of GSH (mg/kg)	Plasma GOT (Absorbance)
PBS (Control)	—	0.892 ± 0.061
Free GSH	81.8	0.729 ± 0.211
PC-liposomes	81.8	0.724 ± 0.156 ^{a)}
PGSH-liposomes/PC	81.8	0.496 ± 0.072 ^{b)}
PGSH-liposomes/CH	81.8	0.751 ± 0.248

^{a)} Significantly different from PBS ($p < 0.05$). ^{b)} Significantly different from PBS ($p < 0.01$). Values are expressed as means ± S.D. of six or seven experiments. The dose of palmitoyl glutathione was expressed as free glutathione. The dose of acetaminophen was 500 mg/kg × 2.

not yet clear how the properties (stability and *in vivo* behavior) of the liposomes described above are related to the species of amino acid or the length of amino acid sequence. This is currently under study.

Transport of Glutathione into the Liver

PGSH-Liposomes accumulate in the liver after intravenous injection as described above. The effectiveness of the liposomes as transporters of glutathione to prevent liver necrosis was examined in terms of their suppressing effect on the increase of plasma GOT induced by a high dose of acetaminophen. The results are expressed as absorbance determined by the method of Reitman and Frankel,²²⁾ as shown in Table IV.

PGSH-Liposomes containing phosphatidylcholine in an equimolar ratio showed remarkable suppression of the increase of plasma GOT ($p < 0.01$). PC-Liposomes containing free glutathione in the aqueous phase were also slightly effective ($p < 0.05$). However, PGSH-liposomes without phosphatidylcholine were not effective ($p > 0.05$). Though it is not clear whether the accumulated PGSH-liposomes in the liver are taken up by the Kupffer cells or parenchymal cells, the results obtained in this experiment suggest that glutathione was transferred into the parenchymal cells. Uptake of small liposomes by the parenchymal cells has been reported,^{12,13)} so the sonicated PGSH-liposomes may be directly taken up by the cells. It is uncertain whether the suppressing effect observed in this study is attributable to acylated glutathione or hydrated free glutathione. However, Anderson *et al.*¹⁰⁾ reported the conversion of the glutathione ester to the free form in the cells, so free glutathione might be the effective agent in this case. Phosphatidylcholine seems to be indispensable for the effectiveness of glutathione or palmitoyl glutathione in liposomal form and it may play an important role in the uptake of the liposomes. A study of the liposome-cell interaction should yield interesting results.

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